

# Distinct roles of mouse laminin $\beta 1$ long arm domains for $\alpha 1\beta 1\gamma 1$ trimer formation

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**Abstract** Mouse embryonal carcinoma F9 cells expressing partial mouse laminin  $\beta 1$  covering either the C-terminal end ( $\Delta\beta 1S$ ) or the whole ( $\Delta\beta 1L$ ) of the long arm were established to study the assembly and interchain disulfide-bonding of  $\beta 1$  to endogenous laminin  $\alpha 1$  and  $\gamma 1$ . Both  $\Delta\beta 1S$  and  $\Delta\beta 1L$  were disulfide-bonded to  $\gamma 1$  but only  $\Delta\beta 1L\gamma 1$  dimer formed a disulfide-bonded  $\alpha 1\Delta\beta 1L\gamma 1$  trimer which was actively secreted into the medium. Meanwhile, in the cells producing  $\Delta\beta 1S\gamma 1$  dimer, the level of endogenous  $\alpha 1\beta 1\gamma 1$  was reduced but the level of monomeric  $\alpha 1$  was increased, suggesting that  $\alpha 1$  was recruited to trimer formation with the  $\Delta\beta 1S\gamma 1$  dimer without disulfide-bonding. This shows that the  $\Delta\beta 1S\gamma 1$  dimer can associate with  $\alpha 1$  but not support the disulfide-bonding at the N-terminus of the long arm of  $\alpha 1$ . While control cells secrete neither monomeric  $\alpha 1$  nor the  $\beta 1\gamma 1$  dimer into the medium, the  $\Delta\beta 1S\gamma 1$  producing cells probably do as  $\alpha 1\Delta\beta 1\gamma 1$  trimer. We thus propose that the N- and C-termini of the long arm of laminin  $\beta 1$  have distinct roles for trimer formation.

**Key words:** Laminin; Heptad repeat; Disulfide-bonding

## 1. Introduction

Laminins constitute a family of basement membrane glycoproteins which affect tissue morphogenesis by their effects on proliferation, migration and differentiation of various types of cells [1–4]. The best studied mouse Engelbreth Holm Swarm (EHS) tumor isoform (laminin-1) is composed of  $\alpha 1$  (400 kDa),  $\beta 1$  (220 kDa) and  $\gamma 1$  (210 kDa) chains, assembled and disulfide-bonded in a cross-shaped structure with three short arms and one rod-like long arm [5–7]. Structural analysis [8] and molecular cloning [9–11] of laminins have identified the long arm to be the site of chain assembly. It has many repeats of a heptad motif with hydrophobic residues at the first and fourth positions and charged residues at the fifth and seventh positions [12]. They form a hydrophobic surface along the  $\alpha$ -helix with two charged edges at both sides. Interchain hydrophobic interactions at this surface drive chain assembly and ionic interactions at the edges determine chain selectivity. In addition,  $\beta 1$  and  $\gamma 1$  are disulfide-bonded to each other at the N- and C-termini of the long arm while  $\alpha 1$  is disulfide-bonded to  $\beta 1$  and  $\gamma 1$  only at the N-terminus of the long arm (Fig. 1, left panel). Since only  $\alpha\beta\gamma$  trimers with the same N- to C-terminus orientation are selectively formed, intracellular assembly of laminin chains is a highly controlled process.

In experiments on de- and re-naturing proteolytic fragments of laminin 1, Engel and coworkers showed that E8 fragments of three chains, which correspond to the C-terminal end of the

long arm, mimic normal intracellular assembly [13,14]. By preparing recombinant or synthetic peptides comprising mouse  $\alpha 1$ ,  $\beta 1$  and  $\gamma 1$  partial sequences, Yamada and coworkers [15–18] showed that chain selection is controlled by defined sites at the C-terminal end of the long arm. Despite these in vitro data, the intracellular process of chain assembly is not well analyzed. Especially, not much is known about the interchain disulfide-bond formation between laminin chains.

In this study, we expressed mouse  $\beta 1$  sequences covering either the C-terminal end or the whole of the long arm in mouse embryonal carcinoma F9 cells and detected disulfide-bond formation between endogenous  $\gamma 1$  and the ectopic  $\beta 1$  mutant. Endogenous  $\alpha 1$  was assembled to this hybrid  $\beta 1\gamma 1$  dimer to form a  $\alpha 1\beta 1\gamma 1$  trimer but it was disulfide-bonded only when the  $\beta 1$  sequence covered the whole long arm. While control cells secrete neither monomeric  $\alpha 1$  nor  $\beta 1\gamma 1$  dimer into the medium, cells producing the hybrid dimer secrete monomeric  $\alpha 1$  probably by forming a  $\alpha 1\beta 1\gamma 1$  trimer without disulfide-bonding between  $\alpha 1$  and the hybrid  $\beta 1\gamma 1$  dimer.

## 2. Materials and methods

### 2.1. Plasmid construction

Fig. 1 shows a simplified structure of laminin-1 and the domains of mouse laminin  $\beta 1$  expressed in F9 cells. The plasmids are based on pEFneoER (provided by Dr. A. Yoshimura) [19], which has erythropoietin receptor (ER) cDNA under the control of the elongation factor (EF)  $1\alpha$  promoter. The signal sequence of ER was used to insert the  $\beta 1$  sequence into the endoplasmic reticulum. For the construction of pEFA $\beta 1S$ , a cDNA fragment corresponding to the  $\beta 1$  sequence [10] of amino acid residues 1540–1765 was prepared with the polymerase chain reaction (PCR) using pLAM [20] as a template and two primers, 5'-GCTGGATCCGCTTGCAGCAGAGTGCAGCTGA-3' and 5'-CGCGAATTCGCTAAGCAGGTGCTGTAAAA-CCG-3'. The ER cDNA fragment in pEFneoER was replaced by this fragment at *Bam*HI and *Eco*RI sites. For the construction of pEFA $\beta 1L$  containing the  $\beta 1$  sequence of amino acid residues 953–1765, a *Hinc*II-*Cla*I fragment of pLAM was inserted between the *Sma*I and *Cla*I sites of pEFA $\beta 1S$ .

### 2.2. Cloning of F9 cells expressing partial $\beta 1$ sequences

Embryonal carcinoma F9 cells were maintained as stem cells [21] and transfected with 20  $\mu$ g of plasmid DNA by calcium phosphate coprecipitation [22]. The *neo*-resistant clones were selected on a medium containing 0.25 mg/ml of G418. More than 10  $\Delta\beta 1L$ -expressing cell lines were established and all gave the same results. However, only one  $\Delta\beta 1S$ -expressing cell line was available in this study.

### 2.3. Metabolic labeling of the cells and immunoprecipitation

The F9 cells in 35 mm dishes were labeled with 600  $\mu$ l of methionine-free Eagle's minimal essential medium (Sigma) containing 0.3 mCi/ml Tran<sup>35</sup>S label (ICN Radiochemical Inc.) for 4 h at 37°C. After removing the medium, the cells were lysed by adding 1000  $\mu$ l of a buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM NaCl, 5 mM EDTA, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and 1 mM phenylmethylsulfonyl fluoride (PMSF). Aliquots of cell lysate corresponding to  $1.5 \times 10^7$  cpm of incorporated radioactivity into cellular

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proteins were diluted to 500  $\mu$ l with the same buffer, and laminin chains were immunoprecipitated overnight at 4°C by mixing 1  $\mu$ l of antiserum against mouse EHS laminin [23,24]. This antiserum immunoprecipitates  $\alpha$ 1 $\beta$ 1 $\gamma$ 1 trimers from various origins and gives stronger signals of  $\beta$ 1 and  $\gamma$ 1 relative to  $\alpha$ 1 in the immunoblotting of mouse laminin-1. Aliquots of labeled medium corresponding to the same size of culture were treated as above for the immunoprecipitation of secreted laminin chains.

#### 2.4. SDS-gel electrophoresis

SDS-gel electrophoresis was carried out as described [23,24]. For one-dimensional electrophoresis under non-reducing condition, 3–10% (w/v) acrylamide was used. In two-dimensional electrophoresis, 4% gel was used for the first dimension under non-reducing condition and 3–10% gel was used for the second dimension under reducing condition. After fixation with a solution containing 10% (v/v) trichloroacetic acid, 30% (v/v) methanol and 10% (v/v) acetic acid for 30 min, the gel was dried and analyzed with a Fuji Film BAS 2000 Image Analyzer.

### 3. Results and discussion

Our previous analyses of laminin assembly in mouse embryonal carcinoma F9 cells [25], mouse 3T3-L1 adipocytes [23] and bovine aortic endothelial cells [24] showed that  $\beta$ 1 and  $\gamma$ 1 are first disulfide-bonded through one pair of cysteines at the N- and C-termini of the long arm. Then  $\alpha$ 1 (or its homologue) associates to  $\beta$ 1 $\gamma$ 1 dimer and is disulfide-bonded to  $\beta$ 1 and  $\gamma$ 1 through two pairs of cysteines at the N-terminus of the long arm. The left panel of Fig. 1 is a simplified model of the structure of laminin-1 to show where these cysteine pairs are. The process of this intracellular chain assembly can be studied by metabolic labeling of the cells with [ $^{35}$ S]methionine/cysteine followed by immunoprecipitation of the cell lysate with anti-laminin antiserum and SDS-gel electrophoresis of the immunoprecipitates under non-reducing condition where

inter-chain disulfide-bonds are preserved. As shown in Fig. 2A,  $\alpha$ 1 $\beta$ 1 $\gamma$ 1 trimer and  $\beta$ 1 $\gamma$ 1 dimer are detected in addition to monomeric  $\alpha$ 1,  $\beta$ 1 and  $\gamma$ 1 in control cells. Chain composition of the trimer and dimer can be confirmed by two-dimensional SDS-gel electrophoresis shown in Fig. 3, in which non-reducing electrophoresis in the first dimension (left to right) is followed by reducing electrophoresis in the second dimension (top to bottom). In this electrophoresis, monomeric proteins come to the diagonal while proteins disulfide-bonded to each other migrate to the area below the diagonal and form a vertical line for each disulfide-bonded complex. Since 4% and 3–10% acrylamide gels were used in Fig. 3 for the first and the second dimension, respectively, monomeric proteins smaller than 150 kDa were not separated in the first dimension and came to the vertical line at the right lower half of the gel. Many weak bands with migration faster than 200 kDa are precipitated from the cell lysates (in Fig. 2A). Since none of them has the reported or calculated molecular mass of endogenous or exogenous laminin chains, and most of them migrate as monomers in the two-dimensional electrophoresis (Fig. 3), we concluded they were precipitated non-specifically. To study the behavior of exogenous  $\beta$ 1 fragments, we focused on bands specifically appeared in transgenic cells.

In the cells expressing  $\Delta\beta$ 1S or  $\Delta\beta$ 1L, exogenous  $\beta$ 1 was recruited to the formation of a hybrid dimer and the band corresponding to the  $\Delta\beta$ 1 $\gamma$ 1 or  $\Delta\beta$ 1 $\gamma$ 1 dimer is detected under non-reducing condition (Fig. 2A, lanes  $\Delta\beta$ 1S and  $\Delta\beta$ 1L). Two-dimensional gel electrophoresis in Fig. 3 confirms that endogenous  $\gamma$ 1 was selected by exogenous  $\beta$ 1 for the formation of disulfide-bonded hybrid dimers of  $\Delta\beta$ 1 $\gamma$ 1 or  $\Delta\beta$ 1 $\gamma$ 1. In the cells expressing  $\Delta\beta$ 1L, the exogenous  $\beta$ 1 was further recruited to the formation of an  $\alpha$ 1 $\Delta\beta$ 1 $\gamma$ 1 trimer (Figs. 2 and 3), showing that the  $\beta$ 1 sequence covering the

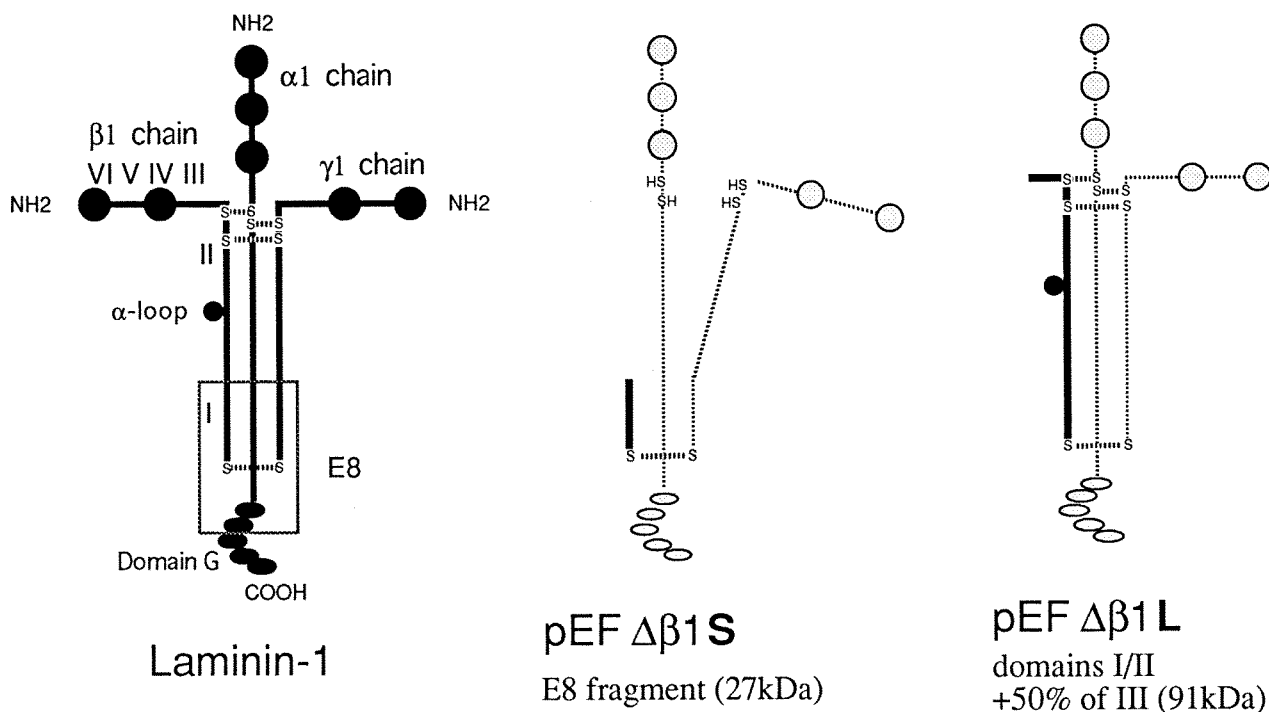


Fig. 1. Plasmids encoding the long arm domain of the mouse  $\beta$ 1 chain. Simplified mouse laminin-1 structure, its major domains and cysteine pairs for interchain disulfide-bonding (S) are shown on the left. The long arm sequences encoded by pEF $\Delta\beta$ 1S and pEF $\Delta\beta$ 1L are shown by thick lines in the middle and on the right, respectively. The domains encoded by each plasmid are briefly explained together with the calculated molecular mass.

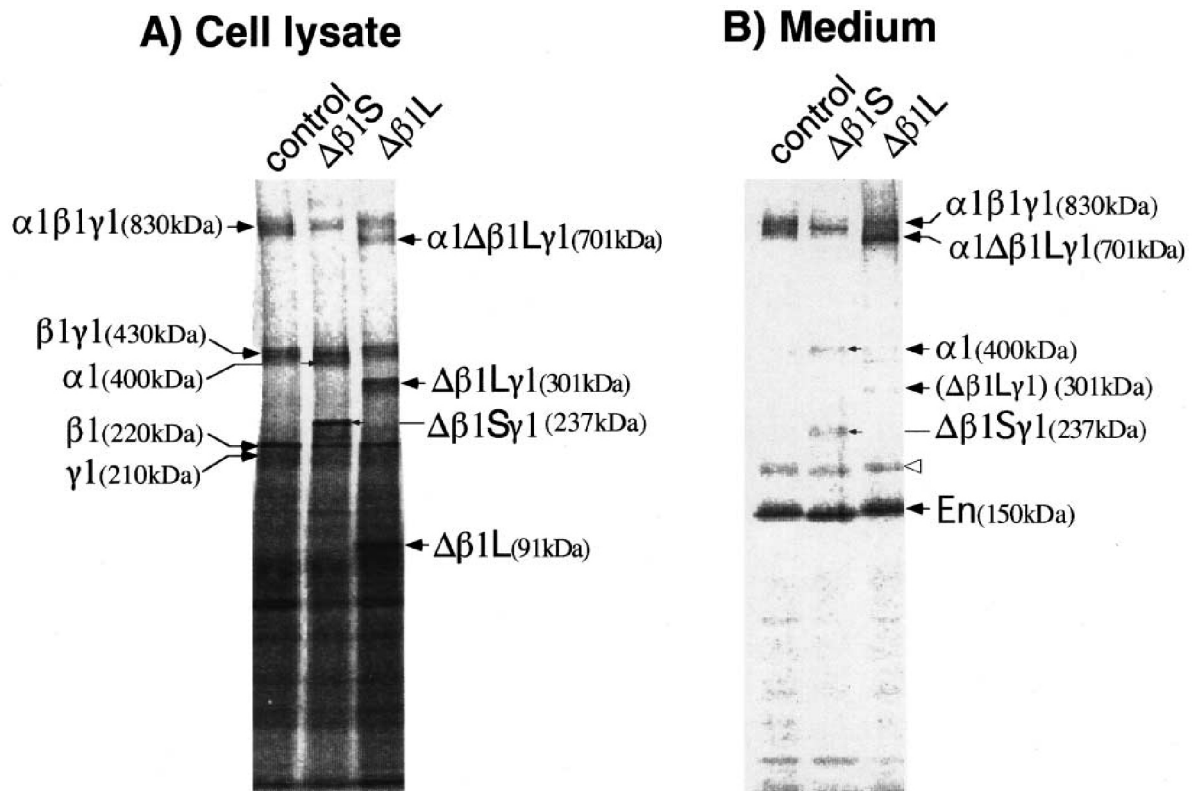


Fig. 2. Non-reducing SDS-gel electrophoresis of immunoprecipitates with anti-mouse laminin-1 antibody from F9 cells expressing partial mouse laminin  $\beta 1$  chain. F9 cells expressing none (control),  $\Delta\beta 1S$  or  $\Delta\beta 1L$  were labeled with [ $^{35}$ S]methionine/cysteine, the cell lysate and medium were immunoprecipitated with anti-mouse laminin-1 antiserum. Immunoprecipitates were separated by SDS-gel electrophoresis under non-reducing condition. Identification of laminin bands is indicated by arrows together with their calculated molecular mass in parentheses. 'En' indicates entactin/nidogen (150 kDa) which has strong affinity to the short arm of  $\gamma 1$  in the  $\alpha 1\beta 1\gamma 1$  trimer. The band indicated by the open arrowhead is an unidentified protein immunoprecipitated even from the control cells.

whole long arm can support the association of endogenous  $\alpha 1$  and its disulfide-bonding to the  $\Delta\beta 1L\gamma 1$  dimer. As endogenous  $\alpha 1\beta 1\gamma 1$  trimer, the  $\alpha 1\Delta\beta 1L\gamma 1$  trimer was actively secreted into

the medium (Fig. 2B, lane  $\Delta\beta 1L$ ). In contrast, the C-terminal long arm sequence of  $\beta 1$  corresponding to the  $\beta 1$  segment of fragment E8 (Fig. 1) could not support the disulfide-bonding

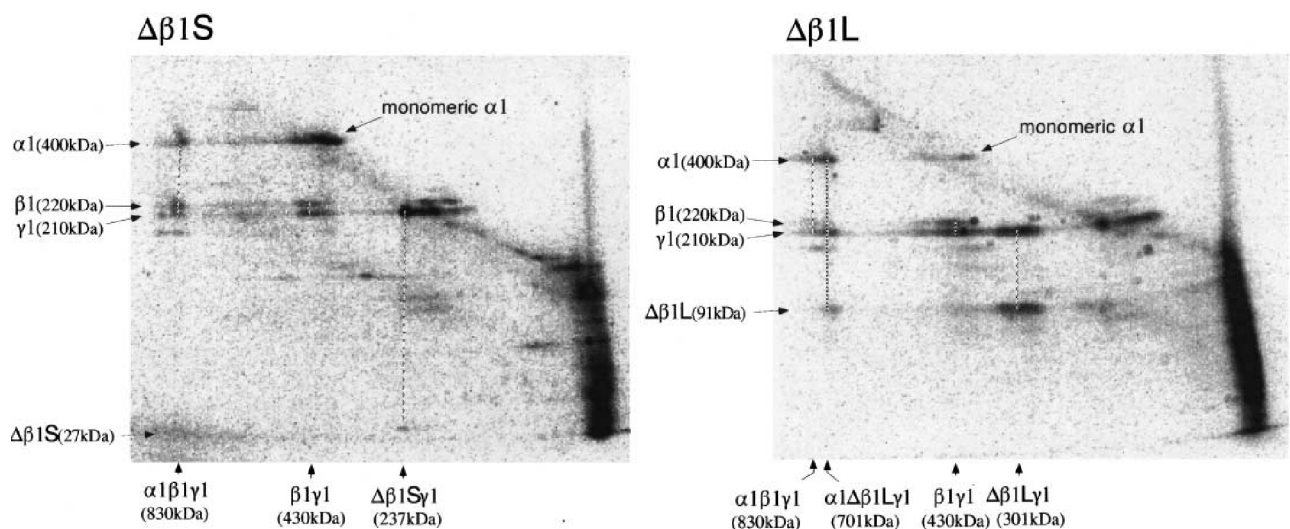


Fig. 3. Two-dimensional electrophoresis of immunoprecipitates with anti-mouse laminin-1 antibody from F9 cells expressing partial mouse laminin  $\beta 1$  chain. Immunoprecipitates from cell lysate prepared as in Fig. 2 were separated by SDS-gel electrophoresis under non-reducing condition in the first dimension (from left to right) followed by reducing condition in the second dimension (from top to bottom). Migration positions of disulfide-bonded complexes in the first dimension and monomers in the second dimension are indicated at the bottom and left margins of each panel, respectively, together with their calculated molecular mass in parentheses. Vertical dotted lines show proteins which are assumed to be disulfide-bonded to each other.

of  $\alpha 1$  to the  $\Delta\beta 1S\gamma 1$  dimer and no band of the hybrid trimer is detected in the cells expressing  $\Delta\beta 1S$  (Figs. 2 and 3). This suggests that a pair of cysteines for interchain disulfide-bonding between  $\alpha 1$  and  $\gamma 1$  is not well oriented without the extension of coiled-coil formation between  $\beta 1$  and  $\gamma 1$  to the N-terminal end of the long arm (Fig. 1, middle panel).

Despite the failure of this disulfide-bond formation, however, the following observations from Figs. 2 and 3 suggest that the  $\Delta\beta 1S\gamma 1$  dimer can associate with  $\alpha 1$  to form the  $\alpha 1\Delta\beta 1S\gamma 1$  trimer. First, the level of endogenous  $\alpha 1\beta 1\gamma 1$  trimer is reduced both in the cell lysate and in the medium of  $\Delta\beta 1S$ -expressing cells when compared with the level in the control cells (Fig. 2). This suggests that one component of the  $\alpha 1\beta 1\gamma 1$  trimer is depleted due to the expression of  $\Delta\beta 1S$ . Second, the level of monomeric  $\alpha 1$  in the cell lysate is increased in  $\Delta\beta 1S$ -expressing cells (Fig. 2A). This fact was not clear in the non-reducing electrophoresis in Fig. 2 due to the poor separation of  $\alpha 1$  from  $\beta 1\gamma 1$ , but the two-dimensional electrophoresis in Fig. 3 undoubtedly shows that monomeric  $\alpha 1$  is specifically increased relative to other spots. SDS-electrophoresis of the same cell lysate under reducing condition showed that the total amount of  $\alpha 1$  remains unchanged regardless of the type of exogenous  $\beta 1$  expressed (result not shown). This result is consistent with the model that a part of  $\alpha 1$  is assembled to the  $\alpha 1\Delta\beta 1S\gamma 1$  trimer without disulfide-bonding to the  $\Delta\beta 1S\gamma 1$  dimer but migrated as a monomer in the electrophoresis under reducing condition. Third, a small amount of monomeric  $\alpha 1$  was secreted into the medium by  $\Delta\beta 1S$ -expressing cells together with the  $\Delta\beta 1S\gamma 1$  dimer (Fig. 2B). In our previous studies [23–25], endogenous laminin chains were allowed to leave the endoplasmic reticulum only after the assembly into disulfide-bonded trimers. Neither monomeric chains nor  $\beta 1\gamma 1$  dimers were transported to the secretory pathway as in the control cells (Fig. 2B). These results suggest that there is a selection mechanism which allows only the  $\alpha\beta\gamma$  trimers to leave the endoplasmic reticulum. Since monomeric  $\alpha 1$  together with the  $\Delta\beta 1S\gamma 1$  dimer is secreted by  $\Delta\beta 1S$ -expressing cells, the C-terminal sequence of  $\beta 1$  corresponding to the  $\beta 1$  segment of fragment E8 is enough to assemble the trimer which can pass through this selection mechanism. Immunoprecipitation of the  $\Delta\beta 1S\gamma 1$  dimer with  $\alpha 1$ -specific antibody might provide solid evidence for trimer formation but an appropriate antibody is not available to us. Although the band was too weak to be identified, an extremely small amount of  $\Delta\beta 1L\gamma 1$  dimer appeared to be secreted also from the cells expressing  $\Delta\beta 1L$  probably together with monomeric  $\alpha 1$  (Fig. 2B). This suggests that a part of the  $\Delta\beta 1L\gamma 1$  dimer failed to be disulfide-bonded to  $\alpha 1$  but secreted as  $\alpha 1\Delta\beta 1L\gamma 1$  trimer as well.

In summary, we demonstrated that the mouse  $\beta 1$  long arm sequence has distinct domains for trimer assembly. Consistent with the results of *in vitro* experiments, we showed here that the C-terminal end of  $\beta 1$  was also enough for the *in vivo* chain selective assembly of the  $\alpha 1\beta 1\gamma 1$  trimer. Our *in vivo* experiment made it possible to analyze the function of the whole long arm for the first time and demonstrated that extension of the coiled-coil formation between  $\beta 1$  and  $\gamma 1$  to the N-terminal end is essential for  $\alpha 1$  to orient itself for the inter-

chain disulfide-bonding to  $\beta 1$  and  $\gamma 1$ . Since  $\alpha 1$ ,  $\beta 1$  and  $\gamma 1$  short arms are combined by three pairs of cysteines at the N-terminal end of the long arm (Fig. 1) and the correct orientation of the short arms is important for the interaction of laminin with cells and other basement membrane components, we propose that the N-terminal domain of the  $\beta 1$  long arm is essential for the organization of functional laminin structure.

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